Supplementary Material

Title: *De novo* variants in *PAK1* lead to intellectual disability with macrocephaly and seizures

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Case Reports

Proband 1 (c.1409T>G, p.(Leu470Arg), *de novo*)

Proband 1 was born via Caesarian section at 29+6 weeks of gestation due to preeclampsia and maternal gestational diabetes. Measurements at birth were normal [weight 1120 g (P25, -0.7 SD), length 41 cm (P67, +0.5 SD), OFC 27.5 cm (P43, -0.2 SD)]. She was in neonatal intensive care for 5 weeks due to respiratory problems and feeding difficulties. A patent ductus arteriosus closed under therapy with Ibuprofen. She had muscular hypotonia and developmental delay. At the corrected age of 5 months macrocephaly became apparent with an OFC of 44.5 cm (>P97, +2.1 SD). Cranial MRI revealed a thin corpus callosum, ventriculomegaly and mildly amorphous hippocampi. MRI FLAIR images are not available. Metabolic workup and genetic testing (chromosomal analysis, array analysis, panel for macrocephaly) were negative. At 21 months she had three febrile seizures. The first febrile seizure was at age 19 months (corrected 16,5 months), she had two more febrile seizures subsequently before the age of 21 months. Durations of the febrile seizures were less than 10 minutes each, respectively and they were reported as independent events. This points towards simple febrile seizures, however, there is no detailed description of the seizures and post-ictal symptoms to classify them in more detail. At age 3 years, epilepsy was diagnosed. Seizures developed to comprise three times status epilepticus, myoclonus, deviation of the eyes and unresponsiveness. She started walking at the age of 3 years. Her neurodevelopmental phenotype was ascertained following published criteria (Zhang *et al.*, 2005). At the age of 3 10/12 years she has global developmental delay / ID with autistic features and is treated for epilepsy with oxcarbazepine and levetiracetam. She has no active speech and communicates non-verbally. She has progressive macrocephaly with a head circumference of 55.5 cm (>P97, +3.86 SD).

Proband 2 (c.397T>C, p.(Ser133Pro), *de novo*)

Proband 2 has been followed over a long period of time. He was born to non-consanguinous parents aged 36 (mother) and 39 (father). He was born at 32 weeks weighing 2268 grams via uncomplicated vertex vaginal delivery after his mother had premature rupture of membranes followed by preterm labor. He remained in the NICU for 4 weeks due to feeding problems. He always had a very large head and at 13 months weight was 10.84 kg, length 76 cm, and head 52 cm (+4 SD) with delayed milestones (sat alone at 11 months, not crawling or standing, no words, some babbling). Paternal OFC was 58.5 cm (+2 SD), maternal OFC was 55.5 cm (50^{th}-75^{th} centile). He walked at approximately 18 months and remained unsteady on his feet with progressive tightness/spasticity that resulted in toe-walking. His MRI scan at age 1 year showed no acute intracranial abnormality, structurally normal-appearing brain, slight ventricular and sulcal prominence without evidence of hydrocephaly. His metabolic PET at age 3 years was normal. He was walking at age 3 but unable to walk at age 12. He developed seizures at age 6 which resulted in motor regression, and EEG (under sedation) revealed high-amplitude paroxysmal discharges at the vertex. His MRI scan at age 6 years showed prominent Virchow-Robin

spaces, small cystic areas of myelomalacia in deep white matter of both cerebral hemispheres. His MRI scan at age 12 years showed dysgenetic appearance of the lateral ventricles with squared off frontal horns, enlarged lateral and third ventricles with borderline to enlarged fourth ventricle and with stretching of the corpus callosum. MRI FLAIR images at age 14 years did not show any informative findings.

When examined at age 13 years his tone was increased in his lower extremities with increased deep tendon reflexes and contracted ankles in extensor posture and muscle wasting in his hands, legs and feet. At age 13 years he had macrocephaly (OFC +5 to +6 SD), hypotonia, moderate hearing loss bilaterally (improved after PE tube placement), bilateral 2-3 toe syndactyly and a history of atonic and tonic-clonic seizures. The seizures were 30-90 seconds in duration and involved upper extremity flexion, lower extremity extension with the eyes open and a tonic posture. Partially, during the seizures he had difficulty breathing and became cyanotic. The frequencies of these episodes were approximately 2 to 3 times a week, with a higher frequency during illness. His developmental delay was profound, remaining non-verbal, showing poor interaction and no walking. His MRI scan at 14 years showed clear megalencephaly, moderate ventriculomegaly with a stretched thin corpus callosum, perivascular white matter signal intensities and, most interestingly, mild cerebellar (and probably also cerebral) atrophy with a very small posterior fossa. In line with this he had shown cerebellar signs of intermittent ataxia. An echocardiogram at age 16 years showed trace mitral regurgitation. At age 17 years, he had just begun to show signs of puberty, and he had severe progressive spastic quadriplegia with markedly tapered distal musculature with hand contractures and equinovarus foot deformity. His OFC was 59.8 cm (mom 57 cm, dad 59.5 cm) and weight 22 kg. He was non-verbal and non-ambulatory, barely pulling himself to stand and showed autistic features like hand flapping. He was unable to cooperate with formal psychometrics testing, however his ID was determined to be profound as he was immobile, non-verbal and unresponsive to environmental stimuli. He was being treated for seizures with clobazam tid and Lacosamide tid, and could only move his head due to progressive spasticity.

Proband 3 (c.361C>T, p.(Pro121Ser), *de novo*)

Proband 3 was born at 34 weeks of gestation, however, the reason of preterm delivery is unknown. Weight at birth was normal. For 4 days he received artificial respiration (CPAP and IPPV) due to infant respiratory distress syndrome (IRDS) and phototherapy for 3 days due to neonatal jaundice. He had muscular hypotonia, attention deficit hyperactivity disorder (ADHD) and developmental delay. At the same time, he developed progressive tremor now comprising positional tremor and tremor of tongue, no intention tremor and no pyramidal signs, however. He is able to walk with mild ataxia. The criteria for classifying his ID were: he attended a school for individuals with pronounced learning disability. IQ was ascertained to be below 55. At age 10 years he spoke first sentences. At age 12 years he could not dress up himself. At 12 years he showed macrocephaly and MRI revealed a thick corpus callosum and non-specific white matter anomalies (see Table 1.). MRI FLAIR images did

not show different abnormalities. MRS showed strongly increased total NAA: N-acetyl aspartate (NAA) in white matter (not in cortex). He had one typical febrile seizure at an uncertain age, CSF analysis was normal and an EEG was not performed.

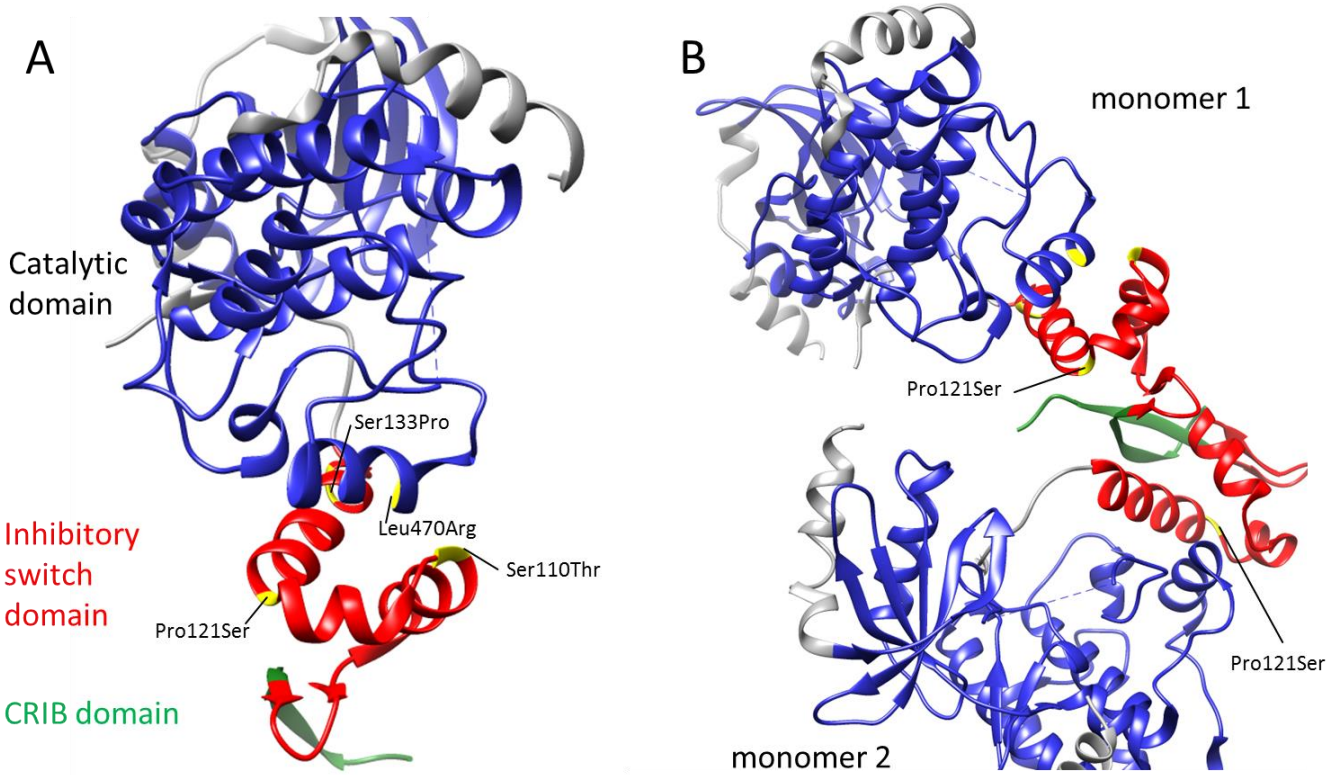
Proband 4 (c.328T>A, p.(Ser110Thr), *de novo*)

Proband 4 was the first child of healthy unrelated parents. He was born with an undescended testicle. At Birth, head circumference was normal (35 cm). At age 1.5 years, he presented with episodes of seizure. Under examination, he presented macrocephaly (OFC: 58 cm), with global developmental delay since 6 months old. Metabolic work-up was negative. Molecular testing for Fragile-X Syndrome was negative as well as skewed X-inactivation, tested for his mother. Testing for chromosomal aberrations via microarray, *PTEN* sequencing and Multiplex ligation-dependent probe amplification (MLPA) were negative. The intellectual disability was determined to be severe as his DQ was below 49 (without access to the formal documentation) and he speaks only few words. He was being treated for seizures with Phenobarbital, Oxcarbazepine, Levetiracetam, Valproate and Clobazam. Because the seizures continued he was referred for a Vagus Nerve Stimulator (VNS) implant. Therafter, the seizures diminished. He is currently treated by three anti-epileptic drugs (AEDs) Valproate, Clobazam and Lacosamide.

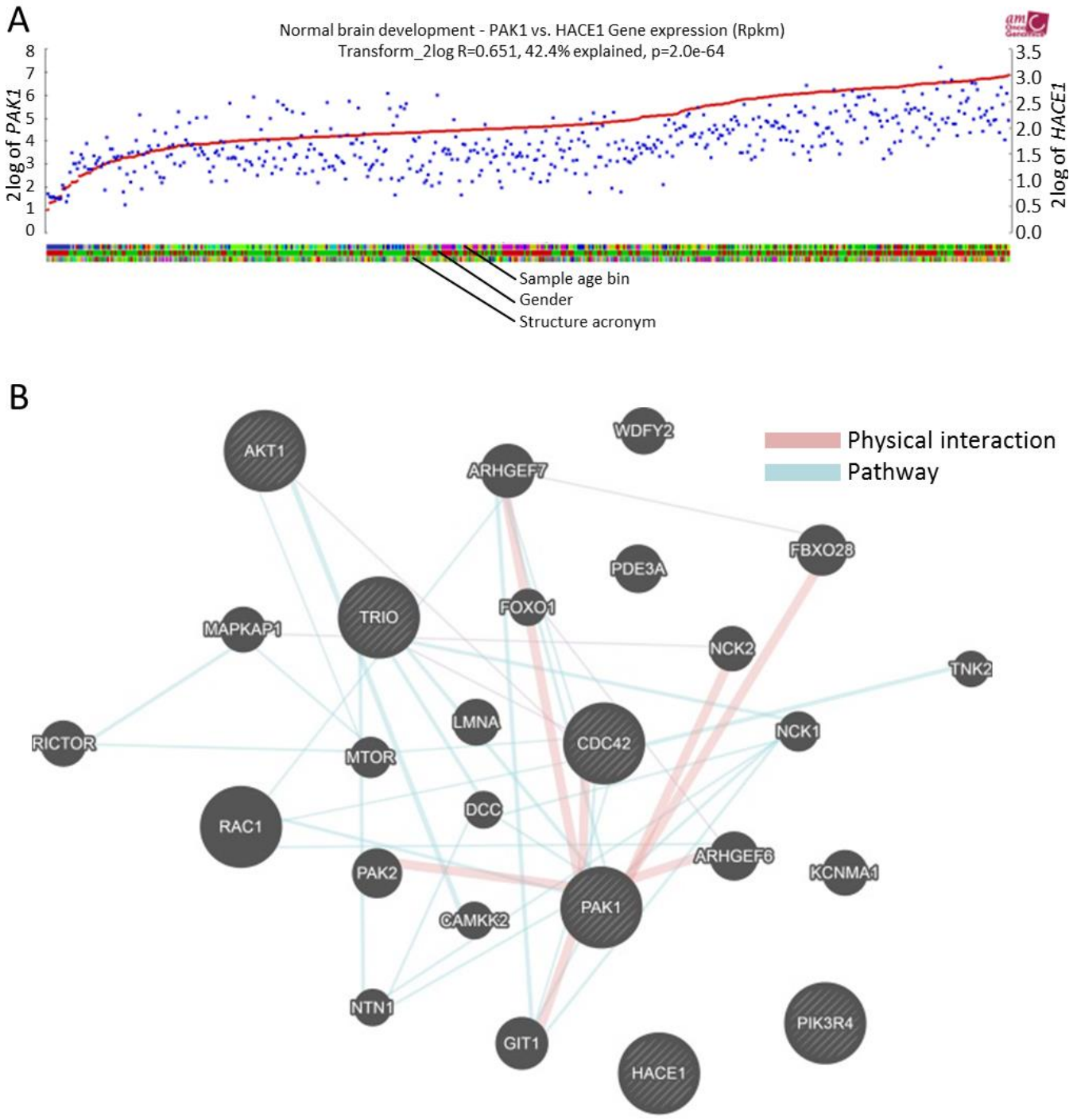
Supplementary Methods

DNA was subjected to exome capture using Agilent SureSelectXT library preparation and human all exon capture (V6, Agilent, Santa Clara, USA) for probands 1 and 2, as well as SeqCap EZ MedExome (Roche, Basel, Switzerland) for proband 3 and the IDT xGen reagent (Integrated DNA Technologies, Coralville, IA, USA) for proband 4. Sequencing was performed on an Illumina NovaSeq6000 (2 x 100 bp, Illumina, San Diego, CA, USA) for proband 1, HiSeq 2500 (2 x 100 bp) for proband 2 and HiSeq 2500 (2 x 125 bp and 2 x 75 bp, respectively) for probands 3 and 4. Sequencing reached a coverage of 20 reads or more by 97.4% (proband 1) and 85% (proband 4) as well as a coverage of 10 reads or more by 86.6% (proband 2) and 95.8% (proband 3) of the targeted bases. Exome sequencing was performed by commercial providers (CeGaT, Tübingen, Germany) for proband 1, ARUP Laboratories (Salt Lake City, Utah) for proband 2, as well as by an academic institution for proband 3 and in collaboration with the Regeneron Genetics Center as previously described for proband 4(Strauss *et al.*, 2018). Bioinformatic processing and filtering was performed using the software Varfeed and Varvis (Limbus, Rostock, Germany) for proband 1, in-house developed software for proband 2 (Varviewer, Salt Lake City, Utah) and proband 3 (Agilent Technologies, Santa Clara, USA) and an RGC developed cloud-based informatics and analytics pipeline for proband 4.

Supplementary Figures



Supplementary Figure 1. Structural analysis of protein changes in PAK1. A. PAK1 monomer with variants Leu470Arg, Ser133Pro and Ser110Thr in the interface between the two major domains (red and blue). **B.** The homodimer of PAK1 builds an asymmetric interface where the Pro121 of only one of the monomers (grey, blue and red) is located in the contact zone of both PAK1 monomers, whereas Pro121 of the other monomer does not (grey, light blue and light red). The change Pro121Ser is also located at a kink between two helices of the auto-inhibitory domain. The structure suggests that this kink is expected to become more flexible in the Ser121 variant.



Supplementary Figure 2. Identification of PAK1 interactors. (A) Co-expression analysis. *HACE1* as an example of the set A of genes correlating with *PAK1* expression (dataset brspv10rs at https://r2.amc.nl), 42.4% explained, p=2.0e-64, R=0.651, Log2 transformed rpkm. (B) Gene set B predicted from set A by network analysis; interacting genes predicted by GENEMANIA (https://genemania.org) based on physical interaction (light red lines), pathway overlap (light blue lines), co-expression, shared protein domains or genetic interactions (no lines shown here for the last two associations). Proteins physically interacting with PAK1 are only partially associated with a neurodevelopmental disorder to date: ARHGEF7, FOXO1, CDC42 (MIM: 616737), NCK2, FBXO28, ARHGEF6 (MIM: 300436), GIT1, RAC1 (MIM: 617751) and PAK2.

Supplementary Tables

Supplementary Table 1: Table of the variants identified in *PAK1*. Variant coding change and affected exons are the same for both *PAK1* transcripts (NM_001128620.1, 16 exons; NM_002576, 15 exons).

	Proband 1	Proband 2	Proband 3	Proband 4
Experiment type	Trio exome sequencing	Trio exome sequencing	Trio exome sequencing	Family exome sequencing
Reference genome	hg19	hg19	hg19	hg19
Variant in exon (of 16)	13	4	4	4
Genotype	chr11:77047135 A>C	chr11:77090328 A>G	chr11:77090364 G>A	chr11:77090397 A>T
cDNA change	c.1409T>G	c.397T>C	c.361C>T	c.328T>A
Amino acid change	p.(Leu470Arg)	p.(Ser133Pro)	p.(Pro121Ser)	p.(Ser110Thr)
Zygosity	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Inheritance	De novo	De novo	De novo	De novo
SIFT	Deleterious, score: 0	Deleterious, score: 0	Deleterious, score: 0	Deleterious, score: 0
CADD	4.75	6.10	5.95	3.60
CADD-PHRED score	26.50	28.70	27.70	25.5
MutationTaster	Disease causing (prob: 1)	Disease causing (prob: 1)	Disease causing (prob: 1)	Disease causing (prob: 1)
Polyphen-2	Probably damaging (score=0.963, sensitivity: 0.78; specificity: 0.95)	Possibly damaging (score=0.956, sensitivity: 0.79; specificity: 0.95)	Probably damaging (score=0.998, sensitivity: 0.27; specificity: 0.99)	Probably damaging (score=0.991, sensitivity: 0.71; specificity: 0.97)
PhyloP (Source: Alamut, range:-14.1 to 6.4)	5.21	3.11	5.69	3.03
GERP score (Source: UCSC genome browser, range: -12.36 to 6.18)	5.91	4.11	5.32	5.32

Supplementary Table 2: Genes and diseases involved in the *PAK1* pathway. PAK1-pathway activation is deduced assuming pathomechanisms depicted in Fig. 3. Decreased cofilin levels may mirror impaired synaptic plasticity. DD. Developmental delay. AR. Autosomal recessive. NA. Not available.

Gene	Role of gene	Associated disorder (MIM#, lead phenotype)	Additional symptoms	Inheritance, pathomechanism	PAK1-pathway presumably	Head phenotype
PAK3	Similar role to PAK1	Mental retardation 30 and 47 (#300558, DD)	moderate to severe intellectual disability, microcephaly, behavioural abnormalities, psychiatric disorder (Allen *et al.*, 1998; Gedeon *et al.*, 2003, 2003; Rejeb *et al.*, 2008)	XLR LOF of PAK3	Deactivated, Increased cofilin	Microcephaly
RAC1	Activates PAK1	Mental retardation 48 (#617751, DD)	brain malformations (Reijnders *et al.*, 2017)	AD LOF and GOF of RAC1	Activated and deactivated, both Increased and decreased cofilin	Macrocephaly (mutation effect unclear) Microcephaly (dominant negative mutation) Normal (activating mutation)
CDC42	Activates PAK1	Takenouchi-Kosaki syndrome (#616737, DD)	growth dysregulation, facial dysmorphism, and neurodevelopmental anomalies (Martinelli *et al.*, 2018; Motokawa *et al.*, 2018; Takenouchi *et al.*, 2015)	AD LOF and GOF of CDC42	Activated and deactivated, both Increased and decreased cofilin	Broad forehead
ARHGEF6	Binds PAK1	Mental retardation 46 (#300436, DD)	X-linked ID with sensorineural hearing loss (Kutsche *et al.*, 2000)	XLR LOF	Deactivated, Increased cofilin	NA
TRIO	Activates RAC1	Mental retardation 44 (#617061, DD)	distinctive facial features, abnormalities of the fingers and microcephaly (Briançon-Marjollet *et al.*, 2008; Ba *et al.*, 2015)	AD LOF of TRIO	Deactivated, Increased cofilin	Microcephaly
HACE1	Aids degradation of RAC1	Spastic paraplegia and psychomotor retardation with or without seizures (#616756, DD)	paraplegia with psychomotor retardation with or without seizures, hypotonia (Hollstein *et al.*, 2015)	AR LOF of HACE1	Activated, Decreased cofilin	Microcephaly, some with large head circumference at birth
LIMK1	Effector of PAK1, inhibits cofilin	Williams-Beuren syndrome (#194050, DD)	deficits in visuospatial cognition(Meng *et al.*, 2002)	AD LOF of LIMK1	Deactivated, Increased cofilin	Macrocephaly and microcephaly
CFL2	Cofilin, Effector of PAK1, enables synaptic plasticity	Nemaline myopathy 7 (#610687, muscular hypotonia)	Kyphoscoliosis (Ockeloen *et al.*, 2012)	AR LOF of CFL2	Downstream of PAK1 Decreased cofilin(Agrawal *et al.*, 2012; Ockeloen *et al.*, 2012)	Normal

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